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Adenovirus-mediated Transfer of a Wild-Type *p53* Gene and Induction of Apoptosis in Cervical Cancer[1]

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ABSTRACT

In most cervical cancers, the function of p53 is down regulated. To explore the potential use of *p53* in gene therapy for cervical cancer, we introduced wild-type *p53* into cervical cancer cell lines via a recombinant adenoviral vector, Ad5CMV-*p53*, and analyzed its effects on cell and tumor growth. The transduction efficiencies of all cell lines were 100% at a multiplicity of infection of 100 or greater. The p53 protein was detected in Ad5CMV-*p53*-infected cells. Protein expression peaked at day 3 after infection and lasted 15 days. The Ad5CMV-*p53*-infected cells underwent apoptosis, and cell growth was greatly suppressed. The Ad5CMV-*p53* treatment significantly reduced the volumes of established s.c. tumors *in vivo*. These results indicate that transfection of cervical cancer cells with the wild-type *p53* gene via Ad5CMV-*p53* is a potential novel approach to the therapy of cervical cancer.

INTRODUCTION

Cervical cancer is the second most common malignancy in women worldwide, accounting for 15% of all cancers diagnosed in women and having an overall 5-year survival rate of 40% (1). Despite aggressive screening with the Papanicolaou smear, this cancer remains an important health problem for women. The most important risk factor for cervical cancer is HPV[3] infection, which has increased the relative risk of developing cervical intraepithelial neoplasia and cervical cancer in case control and cohort studies by 11- to 60-fold (1). Inactivation of the *p53* gene by allelic loss or by point mutation is infrequent in primary cervical cancer, and the overall incidence of point mutations in the *p53* gene in these cancers is 1 to 6% (2).

HPV types 16 and 18 have been identified in up to 90% of cervical cancers (3). The *E6* and *E7* genes of HPV 16 and 18 are frequently coexpressed (4). It has been demonstrated that the E6 and E7 proteins of HPV 16 and 18 are necessary to efficiently immortalize their natural host cells, primary human squamous epithelial cells (5). Furthermore, the continued expression of the *E6* and *E7* regions of the viral genome appears to be necessary for maintaining the malignant phenotype (6–8). It has also been shown that E7 proteins of "high-risk" HPV have an approximately 10-fold higher affinity for Rb than do E7 proteins of "low-risk" HPV (9). High-risk HPV E7 proteins are able to transform established rodent cell lines (10). High-risk HPV E6 proteins promote degradation of p53 via the ubiquitin-dependent protease system (11, 12). This selective degradation of negative regulatory proteins provides a novel mechanism for dominant-acting oncoproteins (13). Howley *et al.* (14) have demonstrated that the inactivation of Rb and p53 is an important step in cervical carcinogenesis. In those studies, HPV-positive cell lines expressed normal levels of Rb and low levels of wild-type p53 (14). The wild-type *p53* gene plays an important role in both the cell cycle and apoptosis. Since wild-type *p53* is theorized to deliver antiproliferative signals, molecular therapy with this gene may be effective against cervical cancer. In other studies, the growth of human NSCLC cells and head and neck squamous carcinoma cells was significantly inhibited *in vitro* and in microscopic tumor models by introducing wild-type *p53* via a recombinant adenoviral vector (15, 16). However, there are no reports on the effects of introduction of a wild-type *p53* gene on cervical cancer cells.

In light of these points, we introduced the wild-type *p53* gene into cervical cancer cells via the recombinant adenoviral vector Ad5CMV-*p53* to determine its effects on the growth of human cervical cancer cells *in vitro* and *in vivo*, and to determine whether the mechanism of this growth-suppressive effect is by induction of apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Eight human cervical cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The HPV and *p53* status of each cell line are shown in Table 1 (17). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum.

Recombinant Adenovirus and Infection. The recombinant *p53* adenoviral vector Ad5CMV-*p53* contains the cytomegalovirus promoter, wild-type *p53* cDNA, and an SV40 polyadenylation signal in a minigene cassette inserted into the *E1*-deleted region of modified adenovirus Ad5 (15). In this study, we used an adenoviral vector containing the same cassette but without the *p53* cDNA (Ad5CMV-*poly A*) as a control. The adenovirus containing the *β-gal* gene, Ad5CMV-*LacZ*, was used to determine transduction efficiencies. Viral stocks were propagated in 293 cells, which are derived from primary embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA. This cell line contains *E1* and is thus highly permissive of the replication of the *E1* replication-deficient adenovirus. Virus was purified from this cell to obtain viral stock. Cells were harvested 36 to 40 h after infection, pelleted, resuspended in PBS, and lysed by three cycles of freezing and thawing. Cell debris was removed by subjecting the lysed cells to CsCl gradient centrifugation. Concentrated virus was dialyzed and stored at −80°C. Infection was carried out by adding the virus to high-glucose DMEM and to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Medium was added, and the cells were incubated at 37°C for the desired length of time. The viral titers were determined by plaque assays (18).

Immunohistochemical Analysis. The Ad5CMV-*p53*-infected cells and injected tissues were analyzed to detect p53 protein expression. The infected cell monolayers were trypsinized, and a cell suspension was put on slides by centrifuging for 5 min at 200 × *g* at room temperature in a cytocentrifuge (Shandon Scientific Co., London, United Kingdom). The slides were air dried and then fixed with 50% ethanol and 50% acetone at −20°C for 20 min. Tissue samples were fixed with 10% formalin for 24 h, and tissue sections were

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[3] The abbreviations used are: HPV, human papillomavirus; Rb, retinoblastoma protein; Tdt, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-biotin nick end labeling; MOI, multiplicity of infection; PFU, plaque-forming unit; β-gal, β-galactosidase; NSCLC, non-small cell lung cancer.

Table 1 *HPV status, p53 status, transduction efficiency[a] and growth inhibition[b] of human cervical cancer cell lines*

Cell line	HPV status	*p53* status	50% transduction efficiency (MOI)	IC_{50} by [^{3}H] count[b]	IC_{50} by cell count
C33A	ND[c]	Mutant (codon 273)	1.2 ± 0.2	9.5 ± 2.2	9.4 ± 2.7
HT3	ND	Mutant (codon 245)	7.5 ± 1.0	63.2 ± 15.2	62.4 ± 11.5
HeLa	HPV 18	wt[d]	10.2 ± 1.7	71.0 ± 10.4	57.2 ± 12.1
C4-I	HPV 18	wt	15.5 ± 1.9	97.0 ± 22.1	112.7 ± 11.2
MS751	HPV 18	wt	15.6 ± 2.6	77.1 ± 9.7	85.6 ± 9.8
ME180	HPV 39	wt	10.7 ± 2.6	138.3 ± 14.3	148.7 ± 22.8
CaSki	HPV 16	wt	1.9 ± 0.8	55.3 ± 13.9	46.6 ± 13.2
SiHa	HPV 16	wt	1.8 ± 0.7	36.7 ± 11.2	25.8 ± 7.4

[a] Determination of transduction efficiency is described in "Results: Adenoviral Infection of Cervical Cancer Cells."
[b] The concentration required for 50% growth inhibition (IC_{50}) of each cell line was determined by [^{3}H]thymidine incorporation and cell count assay after infection with Ad5CMV-*p53* for 6 days. Each IC_{50} is expressed in MOI.
[c] ND, not detected.
[d] wt, wild type.

deparaffinized. Cells and tissue sections were treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed with the Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA). The primary antibody was a mouse anti-human p53 monoclonal antibody DO7 (DAKO Co., Carpinteria, CA), and the secondary antibody was a biotinylated goat anti-mouse IgG (Vector Labs). An avidin and biotinylated horseradish peroxidase macromolecular complex reagent (Vector Labs) was used to detect the antigen-antibody complex. The cells were stained with diaminobenzidine and then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

TUNEL Assay. Apoptotic cells were detected by modification of the previously described TUNEL technique (19). The infected cell monolayers of HeLa cells were trypsinized, and a cell suspension was put on slides by centrifuging for 5 min at 200 × *g* at room temperature in a cytocentrifuge (Shandon). SiHa cells growing on coverslips were treated with 3% H_2O_2 for 15 min at room temperature. The nuclei of the infected cells were stripped from the proteins by incubation with 0.002% protease (Sigma) in PBS for 5 min at 37°C, after which the slides were washed three times in PBS for 5 min. Next, the slides were immersed in TdT buffer [30 mM Tris (pH 7.2), 140 mM sodium cacodylate, and 1 mM cobalt chloride] and covered with TdT (0.1 U/μl; USB, Cleveland, OH) and biotinylated dUTP (0.4 nM; Boehringer Mannheim, Indianapolis, IN) in TdT buffer. The slides were then incubated in a humid atmosphere of 37°C for 60 to 120 min. The reaction was terminated by transferring the slides to termination buffer (300 mM sodium chloride and 30 mM sodium citrate) and incubating for 30 min at room temperature. The slides were then rinsed with PBS, covered with a 2% aqueous solution of BSA for 10 min at room temperature, and rinsed with PBS for 5 min. Next, the slides were incubated with an avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Labs) for 30 min at room temperature, washed three times for 5 min in PBS, and stained with diaminobenzidine for 1 to 2 min at room temperature. Finally, the cells were counterstained with Harris hematoxylin (Sigma).

Western Blot Analysis. Total cell lysates were prepared by lysing cell monolayers in plates with SDS-PAGE sample buffer after rinsing the cells with PBS. Each lane was loaded with 5 μg of cell lysate protein as determined by BCA protein assay (Pierce, Rockford, IL). After electrophoresis at 20 mA for 2 h, the proteins in the gels were transferred to Hybond-ECL membranes (Amersham Corp., Arlington Heights, IL). Then the membranes were blocked with 1% dry milk and 0.1% Tween 20 (Sigma) in PBS and probed with two primary antibodies, mouse anti-human p53 monoclonal antibody DO7 (DAKO) and mouse anti-human actin monoclonal antibody (Amersham), and a secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham). The membranes were developed according to the Amersham ECL protocol. Relative quantities of p53 protein were determined using a densitometer (Molecular Dynamics Inc., Sunnyvale, CA).

Cell Count Assay. Cells were plated at a density of 5 × 10^4 cells/well in 12-well plates. RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum was used as the growth medium. Cells were infected with either Ad5CMV-*p53* or the Ad5CMV-*poly A* viral control. Culture medium alone was used as the mock infection control. Each sample was analyzed in triplicates. After the sixth day of culture, cells were harvested and counted to determine the 50% growth-inhibitory concentration (IC_{50}). Cell viability was determined by trypan blue exclusion.

[^{3}H]Thymidine Incorporation Assay. Cells were also cultured in 96-well, flat-bottomed plates (2 × 10^3 cells/well). Cells were infected with either Ad5CMV-*p53* or Ad5CMV-*poly A* and cultured in RPMI 1640 containing 10% fetal bovine serum for 5 days. Culture medium was used as the mock infection control. Each well was pulse-treated with 0.1 μCi [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; Amersham) for an additional 24 h, after which the cells were harvested with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Individual filter discs were then processed for liquid scintillation counting. The results are presented as the means of triplicate samples.

Tumorigenicity Assay. Cells were infected with Ad5CMV-*p53* and Ad5CMV-*poly A* at a MOI of 20 to 50. An equal amount of cells was treated with medium as a mock infection. Three h after infection, the treated cells were harvested and rinsed twice with PBS. For each treatment, 1 × 10^7 cells in a volume of 100 μl were injected s.c. into female nude (*nu/nu*) mice aged 4–5 weeks (Harlan, Houston, TX). Mice were bred in a defined pathogen-free environment and bore s.c. tumor nodules. Experiments were reviewed and approved by institutional committees for animal care and use and for recombinant DNA research. Seven mice were used for each treatment. The mice were examined every day after injection. Tumor formation and size were evaluated for 44 days. The tumors were measured every other day with calipers in two perpendicular diameters without knowledge of treatment groups. Tumor volume was calculated by assuming a spherical shape, with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

Inhibition of Tumor Growth *in Vivo*. To determine inhibition of tumor growth *in vivo*, Ad5CMV-*p53* was injected into female nude (*nu/nu*) mice (Harlan). In brief, 5 × 10^6 to 1 × 10^7 cells in 100 μl of PBS were injected into the right posterior flank of each mouse through an insulin syringe with a 28 1/2-gauge needle. Seven to 10 animals were used for each group. Three experiments were done: one with a single injection; another with three injections; and a third with six injections. After 20 to 25 days, tumors with a diameter of 5 to 6 mm were established. Either 100 μl of Ad5CMV-*p53* (5 × 10^9 PFUs), Ad5CMV-*poly A* (5 × 10^9 PFUs), or PBS only was injected intratumorally on day 0 in the first experiment; on days 0, 2, and 4 in the second experiment; and on days 0, 1, 2, 3, 4, and 5 in the third experiment. The tumors were measured every other day with calipers in two perpendicular diameters without knowledge of treatment groups. Tumor volume was calculated by assuming a spherical shape, with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

RESULTS

Adenoviral Infection of Cervical Cancer Cells. To determine the adenoviral transduction efficiency of each cell line, cells were infected for 2 days with Ad5CMV-*LacZ*, an adenovirus that expresses the *β-gal* gene. The transduction efficiency was assessed by scoring 500 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-positive cells in each of three replicate dishes and then determining the percentage of β-gal-positive blue cells. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used in the infection. All cell lines inoculated with a single dose of Ad5CMV-*LacZ* at 100 MOI or greater exhibited 100% blue cells (Fig. 1*A*). The 50% transduction efficiencies of this vector in all

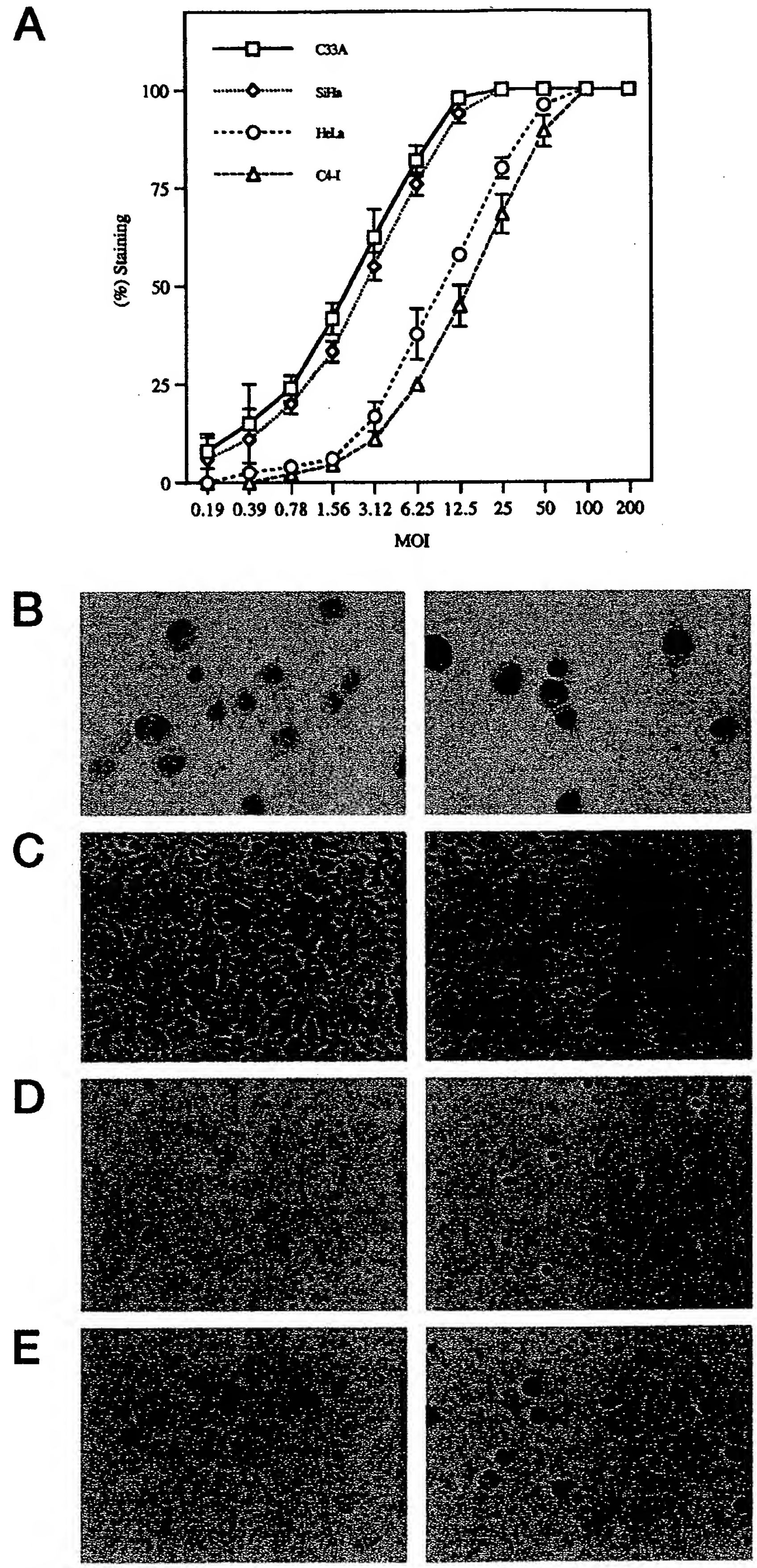

Fig. 1. *A*, representative transduction efficiencies of four human cervical cancer cell lines. A recombinant *β-gal* adenovirus, Ad5CMV-*LacZ*, was used to infect the cells at different MOIs ranging from 0.2 to 200. The percentages of β-gal-positive cells were determined after scoring of 500 cells each on triplicate dishes. *Bars*, SD. *B*, immunohistochemical staining of p53 in SiHa cells. *Left*, 6 h after Ad5CMV-*poly A* infection; *right*, 6 h after Ad5CMV-*p53* infection. ×250. *C*, immunohistochemical staining of p53 protein in SiHa cell tumor. *Left*, 6 h after Ad5CMV-*p53* injection; *right*, 15 days after Ad5CMV-*p53* injection. ×100. *D*, TUNEL staining in SiHa cells. *Left*, 36 h after Ad5CMV-*poly A* infection; *right*, 36 h after Ad5CMV-*p53*-infection. ×100. *E*, TUNEL staining in HeLa cells. *Left*, 36 h after Ad5CMV-*poly A* infection; *right*, 36 h after Ad5CMV-*p53* infection. ×100.

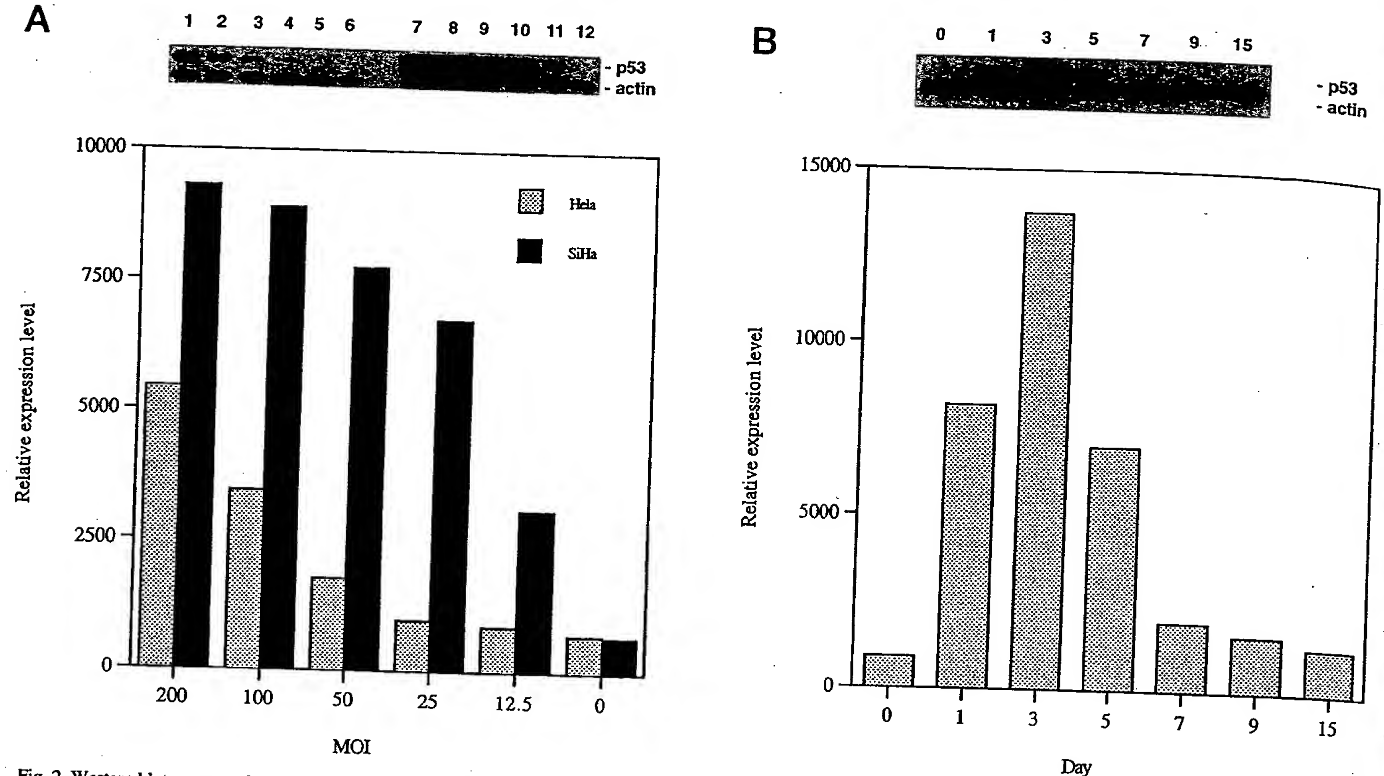

Fig. 2. Western blots were probed with anti-p53 and anti-actin antibodies. *A*, relative levels of p53 protein expression. *Upper*, cellular extracts were subjected to SDS-PAGE (*Lanes 1–6*, HeLa cells; *Lanes 7–12*, SiHa cells). Each cell line was infected for 24 h with Ad5CMV-*p53* at 200 MOI (*Lanes 1* and *7*), 100 MOI (*Lanes 2* and *8*), 50 MOI (*Lanes 3* and *9*), 25 MOI (*Lanes 4* and *10*), 12.5 MOI (*Lanes 5* and *11*), and mock infection (*Lanes 6* and *12*). *Lower*, relative quantities of p53 were determined by densitometry. *B*, time course of p53 expression in SiHa cell. *Upper*, multiple dishes of SiHa cells were infected with Ad5CMV-*p53* at 20 MOI. Cell lysates were prepared at indicated time points after infection. *Lower*, relative quantities of p53 were determined by densitometry.

cervical cancer cell lines (shown in Table 1) varied, but all cell lines showed high transduction efficiencies.

Expression of Exogenous p53 in Cervical Cancer Cells. To determine the expression of the p53 protein in the Ad5CMV-*p53*-infected cervical cancer cell lines, immunohistochemical and Western blot analyses were done using the mouse anti-human p53 monoclonal antibody DO7. Immunohistochemical analysis of SiHa cells infected with Ad5CMV-*p53* revealed characteristic staining of p53 protein in the nucleus 6 h after infection, whereas Ad5CMV-*poly A*-infected cells failed to show p53 staining (Fig. 1*B*). Immunohistochemical analysis of SiHa cell tumors injected with Ad5CMV-*p53* revealed characteristic staining of p53 protein in the nucleus 6 h after injection, and the expression of p53 protein in the Ad5CMV-*p53*-injected tumors lasted 15 days (Fig. 1*C*). Western blot analysis was performed to compare the amount of p53 protein produced following 24 h of infection with Ad5CMV-*p53*. A p53 band, recognized by DO7, was observed in cellular extracts isolated from infected HeLa and SiHa cells. Expression of the p53 protein was highly detectable in SiHa cells compared with HeLa cells. Samples isolated from noninfected HeLa and SiHa cells exhibited very low levels of p53 protein (Fig. 2*A*). The time course of p53 expression in SiHa cells is shown in Fig. 2*B*. Multiple dishes of SiHa cells were infected with Ad5CMV-*p53* at 20 MOI. Following 12 h of incubation, the medium that contained virus was replaced with fresh medium, and each dish was cultured for an additional 2 to 14 days. Cell lysates were then prepared at indicated time points after infection. The p53 protein expression peaked 3 days after infection and began to decline thereafter. However, the intensity of the p53 band isolated from infected SiHa cell lysates at day 15 remained significantly higher than that from noninfected SiHa cells.

Effect f Exogen us p53 n Cervical Cancer Cell Growth. To characterize the apoptotic effects of Ad5CMV-*p53*, the morphological changes in SiHa cells and HeLa cells were examined after infection with Ad5CMV-*p53* at 100 MOI. Within 24 to 48 h after infection, an apparent morphological change occurred, with portions of the cell population rounding up and their outer membranes, forming blebs. These changes are part of a series of histologically predictable events that suggest programmed cell death (16). On the other hand, cells infected with the control adenovirus, Ad5CMV-*poly A*, grew normally with no histomorphological abnormalities. To determine whether the morphological changes seen in the Ad5CMV-*p53*-infected cells were apoptotic processes, SiHa and HeLa cells were stained by the TUNEL method. SiHa and HeLa cells showed apoptotic nuclear DNA fragmentation 36 h after infection with Ad5CMV-*p53* at 100 MOI (Fig. 1, *D* and *E*). The growth of the Ad5CMV-*p53*-infected SiHa cells was greatly suppressed when estimated by cell count and [^{3}H]thymidine incorporation assay 6 days after infection (Fig. 3). The cells were treated with either Ad5CMV-*p53*, Ad5CMV-*poly A*, or medium only. The IC_{50} for all cell lines is shown in Table 1. The IC_{50}, as determined by [^{3}H]thymidine incorporation assay, varied from 10 to 138 MOI. The IC_{50} as determined by cell count assay varied from 9 to 149 MOI among cell lines. The value of each cell growth assay was reproducible in five independent experiments.

Inhibition of Tumorigenicity. To examine whether the Ad5CMV-*p53* virus could inhibit tumorigenicity of human cervical cancer cells, nude mice were injected s.c. with C33A, HT3, HeLa, MS751, and SiHa cells to initiate tumor formation. Each mouse received one injection of 1×10^7 cells that had been infected with Ad5CMV-*p53* or Ad5CMV-*poly A* at 20 to 50 MOI for 3 h. SiHa cells treated with medium alone were used as mock-infected controls. Tumors were formed only from the mock- or control virus-infected cells; mice that received Ad5CMV-*p53*-treated cells did not develop tumors (Fig. 4; Table 2). Tumor ulceration in some animals limited the relevance of

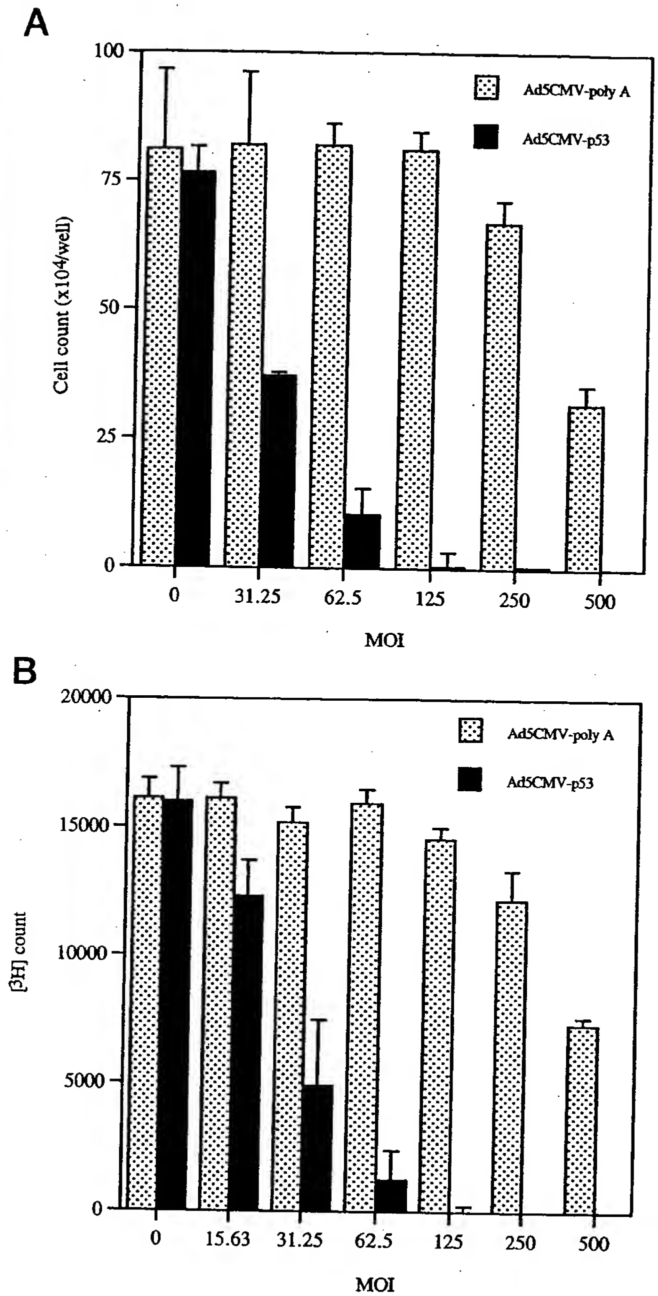

Fig. 3. *A*, inhibition of the growth of SiHa cells as determined by a cell count assay. The cells were inoculated at densities of 5 × 10[4] cells/well in each 12-well plate 24 h before infection. At each indicated point, cells in three wells on each well plate were trypsinized and counted. The mean cell counts for triplicate wells at day 6 after infection were plotted. *Bars*, SD. *B*, inhibition of the growth of SiHa cells by [^{3}H]thymidine incorporation assay. The cells were inoculated at densities of 2 × 10[3] cells/well in each 96-well plate 24 h before infection. At each indicated point, cells of three wells were infected for 5 days, pulse-treated with [^{3}H]thymidine for an additional 24 h, trypsinized, harvested, and counted by liquid scintillation counter. The mean cpm for triplicate wells were plotted. *Bars*, SD.

tumor size measurements after day 46. This study was initiated with seven mice per group; no mice failed to complete the study. Furthermore, Ad5CMV-*p53* also completely inhibited tumorigenicity of C33A, HT3, HeLa, and MS751 cells (Table 2).

Inhibition of Tum r Growth *in Vivo*. To address the feasibility of *p53* gene therapy for established tumors, the efficacy of Ad5CMV-*p53* in inhibiting tumor growth was evaluated in a tumor-bearing nude mouse model using human cervical cancer C33A, HT3, MS751, and SiHa cells. Tumors were allowed to grow for 20 to 25 days to a diameter of 5 to 6 mm. In the first experiment, mice then received one intratumoral injection of PBS only, Ad5CMV-*poly A*, or Ad5CMV-*p53* on day 0. In the mice treated with PBS only or with Ad5CMV-*poly A*, SiHa cell tumors continued to grow rapidly throughout the treatment, whereas growth was greatly reduced for 21 days in the tumors treated with the Ad5CMV-*p53*, then tumors began to regrow (Fig. 5*A*). The single injection of Ad5CMV-*p53* reduced tumor size by 62% 30 days after infection compared with the single injection of PBS only, whereas the single injection of Ad5CMV-*poly A* did not reduce tumor size significantly (Table 3).

In the second experiment, mice received three intratumoral injections of PBS only, Ad5CMV-*poly A*, or Ad5CMV-*p53* on days 0, 2, and 4. In the mice treated with PBS only or Ad5CMV-*poly A*, SiHa cell tumors continued to grow rapidly throughout the treatment, whereas those treated with Ad5CMV-*p53* grew at a greatly reduced

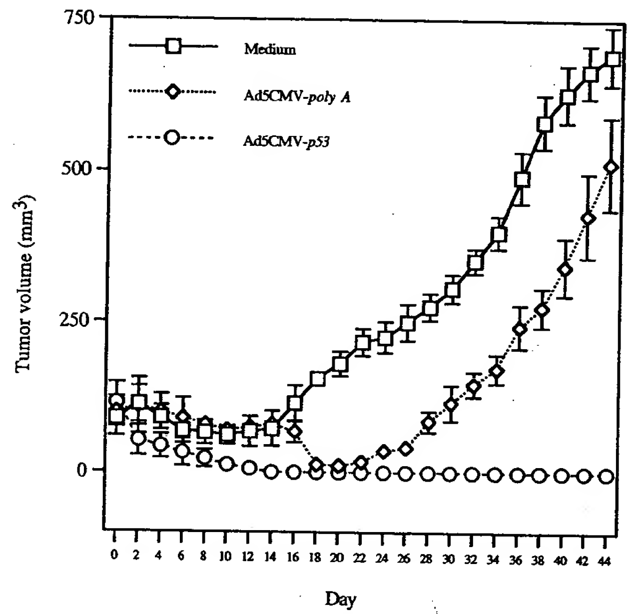

Fig. 4. Effect of pretreatment with Ad5CMV-*p53* on tumorigenicity of SiHa cells in nude mice. SiHa cells were treated with Ad5CMV-*p53* or Ad5CMV-*poly A* at 50 MOI or medium only for 3 h. The treated cells were injected s.c. at 1 × 10[7] cells/mouse. Tumor size was determined every other day after injection. *Points*, means. *Bars*, SD.

Table 2 *Effect of Ad5CMV-p53 on tumorigenicity in nude mice*

Cell line	Treatment	No. of tumors/ no. of mice	Mean volume mm^3 ± SD	(%)
C33A[a]				
	PBS	7/7	1678 ± 839	(100)
	Ad5CMV-*poly A*	5/7	1055 ± 274	(62.9)
	Ad5CMV-*p53*	0/7	0 ± 0	(0)
HT3[a]				
	PBS	7/7	296 ± 145	(100)
	Ad5CMV-*poly A*	6/7	196 ± 67	(66.2)
	Ad5CMV-*p53*	0/7	0 ± 0	(0)
HeLa[b]				
	PBS	7/7	343 ± 136	(100)
	Ad5CMV-*poly A*	7/7	238 ± 43	(69.4)
	Ad5CMV-*p53*	0/7	0 ± 0	(0)
MS751[b]				
	PBS	7/7	1378 ± 839	(100)
	Ad5CMV-*poly A*	7/7	1270 ± 266	(92.2)
	Ad5CMV-*p53*	0/7	0 ± 0	(0)
SiHa[a]				
	PBS	7/7	692 ± 48	(100)
	Ad5CMV-*poly A*	6/7	514 ± 77	(74.3)
	Ad5CMV-*p53*	0/7	0 ± 0	(0)

[a] Cells were infected with Ad5CMV-*p53* at 20 MOI for 3 h. The treated cells were injected s.c. at 1 × 10[7] cells/mouse. Tumor sizes were determined 44 days after injection.
[b] Cells were infected with Ad5CMV-*p53* at 50 MOI for 3 h. The treated cells were injected s.c. at 1 × 10[7] cells/mouse. Tumor sizes were determined 44 days after injection.

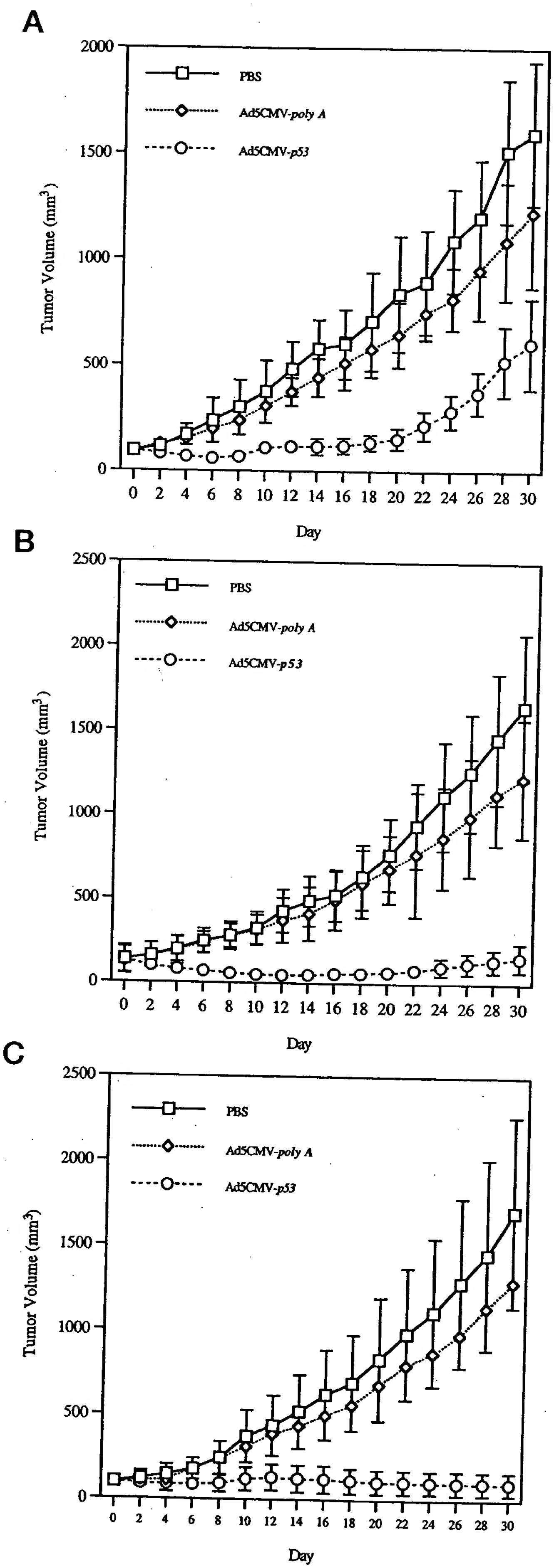

Table 3 *Effect of Ad5CMV-p53 on established in vivo tumor growth in nude mice*

Cell line	Treatment	No. of tumors/ no. of mice	Mean volume $mm^3 \pm SD$	(%)
Experiment I[a]				
SiHa	PBS	7/7	1613 ± 340	(100)
	Ad5CMV-*poly A*	7/7	1224 ± 352	(75.9)
	Ad5CMV-*p53*	7/7	605 ± 215	(37.5)
Experiment II[b]				
SiHa	PBS	7/7	1647 ± 432	(100)
	Ad5CMV-*poly A*	7/7	1324 ± 350	(80.4)
	Ad5CMV-*p53*	7/7	129 ± 3	(7.8)
Experiment III[c]				
C33A	PBS	7/7	2110 ± 548	(100)
	Ad5CMV-*poly A*	7/7	1764 ± 722	(83.6)
	Ad5CMV-*p53*	5/7	82 ± 45	(3.9)
HT3	PBS	7/7	666 ± 328	(100)
	Ad5CMV-*poly A*	7/7	406 ± 348	(61.0)
	Ad5CMV-*p53*	2/7	25 ± 12	(3.8)
MS751	PBS	7/7	1593 ± 491	(100)
	Ad5CMV-*poly A*	7/7	1398 ± 233	(87.8)
	Ad5CMV-*p53*	6/7	192 ± 96	(12.1)
SiHa	PBS	10/10	1959 ± 561	(100)
	Ad5CMV-*poly A*	10/10	1589 ± 393	(81.1)
	Ad5CMV-*p53*	8/10	95 ± 45	(4.8)

[a] Ad5CMV-*p53* (5×10^9 PFUs/100 μl) was injected into the tumor on day 0. Tumor sizes were measured 30 days after injection.
[b] Ad5CMV-*p53* (5×10^9 PFUs/100 μl) was injected into the tumor on days 0, 2, and 4. Tumor sizes were measured 30 days after the first injection.
[c] Ad5CMV-*p53* (5×10^9 PFUs/100 μl) was injected into the tumor days 0, 1, 2, 3, 4, and 5. Tumor sizes were measured 30 days after the first injection.

rate (Fig. 5*B*). Thirty days after the initial injection, Ad5CMV-*p53* had reduced tumor size by 92% compared with PBS only, whereas Ad5CMV-*poly A* did not significantly reduce tumor size. This result was reproducible in three repeated experiments (Table 3).

In the third experiment, mice received six intratumoral injections of PBS only, Ad5CMV-*poly A*, or Ad5CMV-*p53* on days 0, 1, 2, 3, 4, and 5. In the mice treated with PBS only or Ad5CMV-*poly A*, SiHa cell tumors continued to grow rapidly throughout the treatment, whereas tumor growth in mice treated with Ad5CMV-*p53* was greatly reduced (Fig. 5*C*). Six injections of Ad5CMV-*p53* reduced the size of SiHa cell tumors by 95% compared with six injections of PBS only, whereas six injections of Ad5CMV-*poly A* did not reduce significantly the size of SiHa cell tumors. Two of 10 tumors injected with Ad5CMV-*p53* showed pathologically complete tumor regression of SiHa cell tumors. Six injections of Ad5CMV-*p53* also showed similar growth suppressive effects: 96% in C33A cell tumors, 96% in HT3 cell tumors, and 88% in MS751 cell tumors (Table 3). Six injections of Ad5CMV-*p53* induced pathologically complete tumor regression in 2 of 7 C33A cell tumors, 5 of 7 HT3 cell tumors, and 1 of 7 MS751 cell tumors. There was no significant difference in the body weights of any of the mice treated in these experiments. Together, these results indicate that Ad5CMV-*p53* can inhibit tumor growth of human cervical cancer cells *in vivo*. No animals died during these experiments.

Fig. 5. Effects of treatment with Ad5CMV-*p53* on tumor growth of SiHa cells in nude mice. *A*, effect of single injection of Ad5CMV-*p53* on tumor growth of SiHa cells in nude mice. Mice were injected s.c. with 5×10^6 SiHa cells/mouse. Twenty-five days later, 100 μl of Ad5CMV-*p53* (5×10^9 PFUs), Ad5CMV-*poly A* (5×10^9 PFUs), or PBS alone was injected into tumors of 5 to 6 mm in diameter. A single intratumoral injection was performed on day 0. Seven mice were used for each treatment group. *Points*, means. *Bars*, SD. *B*, effect of three injections of Ad5CMV-*p53* on tumor growth of SiHa cells in nude mice. Mice were injected s.c. with 5×10^6 SiHa cells/mouse. Twenty-five days later, 100 μl of Ad5CMV-*p53* (5×10^9 PFUs), Ad5CMV-*poly A* (5×10^9 PFUs), or PBS alone was injected into tumors of 5 to 6 mm in diameter. Intratumoral injections were performed on days 0, 2, and 4. Seven mice were used for each treatment group. *Points*, means. *Bars*, SD. *C*, effect of six injections of Ad5CMV-*p53* on tumor growth of SiHa cells in nude mice. Mice were injected with 5×10^6 SiHa cells. Twenty-five days later, 100 μl of Ad5CMV-*p53* (5×10^9 PFUs), Ad5CMV-*poly A* (5×10^9 PFUs), or PBS alone was injected into tumors with a diameter of 5 to 6 mm. Intratumoral injections were made on days 0, 1, 2, 3, 4, and 5. Ten mice made up each treatment group. *Points*, means. *Bars*, SD.

DISCUSSION

Use of a recombinant *β-gal* adenovirus, Ad5CMV-*LacZ*, allowed us to establish the gene transfer efficiency in cervical cancer cells. At more than 100 MOI, all cervical cancer cells were positive for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. The transduction efficiency we observed was almost the same as the human NSCLC cell lines (15). The *p53* gene is wild type in six HPV-positive cell lines used in this study. The p53 protein of these cell lines was barely detected in untransfected cells. The low level of p53 protein in the HPV-positive cell lines has been ascribed to the E6-activated ubiquitin-dependent protease digestion (2). Western blot and immunohistochemical analyses demonstrated that production of the p53 protein was significantly increased in Ad5CMV-*p53*-infected cells when compared with control cells, suggesting that the exogenous *p53* mRNA may be efficiently translated. Time course protein expression studies have shown a peak in protein expression 3 days after infection and a progressive decline to levels still detectable by Western blotting on day 15. These results were similar to those of the previous study of NSCLC cell lines (15). Human cervical cancer cells have a functional genotype equivalent to that of cells in which the *p53* gene product is inactivated by the complexing of the E6 protein with the p53 protein. The adenoviral vector is capable of mediating high levels of p53 expression, which are apparently sufficient to overcome the capabilities of the endogenous E6 to bind the p53 protein.

The *in vitro* growth of the cervical cancer cells we transduced with the wild-type *p53* gene was significantly inhibited when compared with mock-infected and Ad5CMV-*poly A*-infected cells, suggesting that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to the arrest of the cell cycle at G_1 (20), apoptosis (21), or induction of another tumor suppressor gene, such as *WAF1/CIP1* (22). The induction of apoptosis is one of several documented functions of wild-type p53. Apoptosis is a selective process of physiological cell deletion. It also seems that chromatin cleavage is the most characteristic biochemical feature of the process. Its gross features include nuclear chromatin condensation, compactness of cytoplasmatic organelles, and the appearance of pedunculated protuberances on the cell surface (19). To determine the role of apoptosis in the growth inhibition we observed, we used the TUNEL method, which is based on the specific binding of TdT to 3′-OH ends of DNA and the consequent synthesis of a polydeoxynucleotide polymer (19). Our TUNEL results showed apoptotic nuclear DNA fragmentation in the Ad5CMV-*p53*-infected cells, which strongly suggests that the mechanism of growth suppression and cell death induced by Ad5CMV-*p53* in cervical cancer cells is apoptosis.

Overexpression of p53 protein in cervical cancer cells completely suppressed the tumorigenicity of the *p53*-mutated C33A and HT3 cell lines, the HPV 18-positive MS751 and HeLa cell lines, and the HPV 16-positive SiHa cell line in nude mice. Adenovirus-*p53* also inhibits the tumorigenicity of a *p53*-deleted osteosarcoma cell line and a *p53*-deleted NSCLC cell line (15, 23), but there are no reports of the effect of exogenous wild-type *p53* on the tumorigenicity of mutated and wild-type *p53* cell lines. In this report, we demonstrated that *p53* tumor suppressor gene therapy can suppress the tumorigenicity of a *p53*-mutated cell line and that overexpression of p53 protein is enough to overcome p53 protein inactivation by the HPV E6 oncoprotein to suppress tumorigenicity of HPV 16- and HPV 18-positive cervical cancer cells. Moreover, overexpression of p53 protein in cervical cancer cells efficiently reduced tumor growth in nude mouse models. When compared with PBS alone and Ad5CMV-*poly A*, Ad5CMV-*p53* strongly suppressed tumor growth. A single injection of Ad5CMV-*p53* significantly suppressed SiHa cell tumor growth during 21 days and then started to regrow. p53 protein expression in the Ad5CMV-*p53*-injected tissue was still detectable by immunohistochemical analysis on day 15. The duration of this tumor growth suppression by a single injection of Ad5CMV-*p53* is consistent with that of p53 protein expression *in vitro* and *in vivo*. Furthermore, three injections of Ad5CMV-*p53* in the first week suppressed 92% of SiHa cell tumor growth for 30 days. Six injections of Ad5CMV-*p53* in the first week suppressed 88 to 96% of C33A, HT3, MS751, and SiHa cell tumors growth for 30 days and induced complete tumor regression in 14 to 71% of C33A, HT3, MS751, and SiHa cell tumors.

Microscopic tumor models, in which adenovirus-*p53* is injected into the tumor 2 to 4 days after cancer cell inoculation, have been developed in wild-type *p53* and *p53*-mutated head and neck cancer and in *p53* mutated NSCLC (15, 16). However, these tumor models are too small to evaluate and predict the effectiveness of adenovirus-*p53* in humans. A larger established tumor model of 5 to 6 mm in diameter has been developed in the *p53*-deleted SCLC; eight injections of adenovirus-*p53* for 4 weeks significantly suppressed tumor growth (23). Adenovirus induces strong immunogenicity, as several studies have shown. In one, titers of anti-adenovirus antibody rose in the rhesus monkey plasma within 2 weeks after the first infection (24). In another, CD8 immunoreactive cells were absent in adenovirus-infected liver at 3 days but were abundant in adenovirus-infected liver at 7 days (25). In addition, adenovirus-mediated *LacZ* transgene expression is strongly suppressed 14 days after injection of adenovirus-*LacZ* (25). From these facts, it is suggested that adenovirus-mediated transgene expression may be suppressed by 1 to 2 weeks after injection. Nevertheless, our new model of gene therapy for cervical cancer by adenovirus-*p53* is promising, because six injections of adenovirus-*p53* in the first week induced significant tumor reduction and complete tumor regression. However, a syngenic mouse model system should be developed to estimate these growth-inhibitory effects by adenovirus-*p53*.

Despite aggressive screening with the Papanicolaou smear and new therapeutic initiatives using surgery, radiotherapy, and chemotherapy for decades, the survival rate of cervical cancer still remains at 40% worldwide (1). Since the incidence of locoregional recurrence is the highest among the first recurrent sites of cervical cancer (26), the prevention and the treatment of locoregional recurrence is still an important problem in the therapy of cervical cancer. However, a locally advanced tumor of cervical cancer can be easily exposed by vaginal speculum and easily injected with multiple direct doses of adenovirus-*p53*. Therefore, our findings of the significant tumor reduction in HPV 16-positive, HPV 18-positive, and *p53*-mutated cervical cancer cell lines by adenovirus-*p53* seem to provide a sound basis for future clinical trials of cervical cancer gene therapy to improve the prognosis of cervical cancer.

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